Circulating clonotypic B cells in multiple myeloma and monoclonal gammopathy of undetermined significance

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ABSTRACT

The B-cell compartment in which multiple myeloma stem cells reside remains unclear. We investigated the potential presence of mature, surface-membrane immunoglobulin-positive B lymphocytes clonally related to the tumor bone marrow plasma cells among different subsets of peripheral blood B cells from ten patients (7 with multiple myeloma and 3 with monoclonal gammopathies of undetermined significance). The presence of clonotypic immunoglobulin heavy chain gene rearrangements was determined in multiple highly-purified fractions of peripheral blood B-lymphocytes including surface-membrane IgM$^+$ CD27$^-$ naïve B-lymphocytes, plus surface-membrane IgG$^+$, IgA$^+$ and IgM$^+$ memory CD27$^+$ B cells, and normal circulating plasma cells, in addition to (mono)clonal plasma cells, by a highly-specific and sensitive allele-specific oligonucleotide polymerase chain reaction directed to the CDR3 sequence of the rearranged *IGH* gene of tumor plasma cells from individual patients. Our results showed systematic absence of clonotypic rearrangements in all the different B-cell subsets analyzed, including M-component isotype-matched memory B-lymphocytes, at frequencies <0.03 cells/μL (range: 0.0003-0.08 cells/μL); the only exception were the myeloma plasma cells detected and purified from the peripheral blood of four of the seven myeloma patients. These results indicate that circulating B cells from patients with multiple myeloma and monoclonal gammopathies of undetermined significance are usually devoid of clonotypic B cells while the presence of immunophenotypically aberrant myeloma plasma cells in peripheral blood of myeloma patients is a relatively frequent finding.

Introduction

A major challenge in the pathogenesis of both multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS) has been the identification of the cell of origin of both neoplasias. Monoclonal plasma cells (M-PC) from patients with MM usually show a low proliferative capacity as assessed by labeling indices of <0.5%;[1] this finding supports the notion that pre-PC compartments may contain the proliferative M-PC progenitor. In line with this hypothesis, circulating peripheral blood cells clonally identical to bone marrow M-PC have been recurrently detected in MM.[2-7] Apart from the presence of the malignant *IGH* gene rearrangement, these peripheral blood cells have also been found in some patients to carry karyotypic alterations such as trisomy 11, del(17p)-, and oncogenic proteins (e.g. IGH-FGFR3, IGH-MMSET) expressed by CD138$^+$ M-PC.[6-8] Moreover, intraclonal heterogeneity, as assessed through the analysis of the *IGHV* gene sequence[9] or through the investigation of cytogenetic alterations[10] and gene mutations evaluated by whole exome sequencing,[11] has been reported within the M-PC population, suggesting that the tumor PC compartment could be continuously repopulated by more than one stem cell.

There is a growing body of evidence showing the presence of circulating aberrant PC not only in patients with MM but also in those with MGUS,[12-16] and recent studies have demonstrated the presence of cells sharing stem cell properties within the compartment of M-PC.[17] However, the question that remains to be answered is whether or not clonotypic cells are also identifiable in peripheral blood B-cell subsets, which would be the "earliest clonotypic cells". Clonotypic cells were first identified in a sorted CD19$^+$ fraction[2] and the pattern of somatic hypermutation in the VH regions of the *IGH* gene suggested that the malignant cell had passed through the germinal center.[18] This was further supported by the detection of peripheral blood clonotypic memory B cells in MM patients[2,6] and the finding that *in vitro* clonogenicity was reduced when memory B cells were removed.[19] In contrast, screening for cells with a pre-PC phenotype in cell lines,[4,19-21] and analysis of the engraftment of peripheral blood B cells from MM patients into immunodeficient mice[3,19,22,23] have provided inconsistent findings. In parallel, depletion of B cells by rituximab therapy has not shown clear beneficial clinical effects in MM, except in a small fraction of patients with CD20$^+$ PC,[24,25] and evaluation of the clonal hierarchy in light chain-secreting myeloma did not detect clonotypic circulating cells, except in one patient showing peripheral blood infiltration by malignant PC.[26] One explanation for these discrepant results is a simple technical pitfall: the B cells ana-

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lyzed could be contaminated in some cases by circulating PC. In fact, to the best of our knowledge, no highly sensitive molecular analysis of different compartments of highly purified circulating B cells specifically devoid of contaminating circulating M-PC has been performed so far, to confirm or rule out the presence of clonotypic B cells in MM and MGUS.

Here, we investigated the presence of circulating B cells which would be clonally related to the M-PC in patients with MM and MGUS, using an allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) technique aimed at sensitive and specific detection of *IGHVDJ* monoclonal gene sequences unique to the tumor cells of individual patients. This analysis was performed in multiple, different, highly-purified, maturation-associated subsets of peripheral blood B cells ($_{Sm}IgM^+/CD27^-$ naïve, isotype non-switched $_{Sm}IgM+$ and isotype-switched $_{Sm}IgG^+$ and $_{Sm}IgA^+$ memory B-lymphocytes, and PC) from a group of seven patients with MM and three with MGUS.

Methods

Patients and samples

Ten patients (7 with MM and 3 with MGUS) diagnosed with monoclonal gammopathies[27] were studied. In all cases, paired bone marrow and peripheral blood samples were collected at diagnosis for further immunophenotypic characterization, isolation, and molecular analysis of M-PC and other B-cell subsets, after informed consent was given by each individual. The study was approved by the Ethics Committee of the Cancer Research Center of the University of Salamanca, (Salamanca, Spain) and it followed the Helsinki Declaration protocol. The most relevant clinical and biological characteristics of the patients studied are summarized in *Online Supplementary Table S1*.

Multiparameter flow cytometry immunophenotypic studies and cell purification

Multiparameter flow cytometry immunophenotypic studies were performed as recently described[28-31] (and detailed in the *Online Supplementary Methods*) in both bone marrow and peripheral blood samples. Fluorescence-activated cell sorting (FACS) was used to purify bone marrow M-PC as well as peripheral blood B-cell subsets and normal PC (N-PC); whenever present, circulating M-PC were also purified. The purity of the sorted M-PC and the multiple peripheral blood normal B-cell subsets was systematically ≥98%, and only those peripheral blood B-cell fractions which showed no contamination (<0.01%) by circulating peripheral blood M-PC were included in the study; all other fractions were not tested (Table 1).

Analysis of IGH gene rearrangements of bone marrow monoclonal plasma cells and CDR3 sequencing

The malignant *IGH* gene rearrangement was identified in genomic DNA according to the BIOMED-2 protocol[32,33] slightly modified to amplify the specific monoclonal complete VH-JH and incomplete DH-JH. PCR amplified products were identified by high-resolution capillary electrophoresis in an automated ABI PRISM 3130 Avant sequencer, using GENEMAPPER 3.1 software (AB). Clonal PCR products were purified, sequenced and identified as previously reported.[34] Once the segments had been identified, the N-region was highlighted for ASO-primer design due to its high specificity for each individual rearrangement, as previously described.[35]

A qualitative ASO-PCR with the CDR3 specific primer and its respective 6-FAM-labeled *IGH* JH primer, was carried out on purified bone marrow M-PC from each patient; purified peripheral blood B cells from healthy donors were studied in parallel as negative controls. If a single (clonal) peak was detected in a sample, it was sequenced to confirm that the sets of primers were really specific. In order to assess the sensitivity of each patient-specific set of primers used in the ASO-PCR assay (e.g.: the lowest number of cells detected), ASO-PCR analyses were carried out in multiple tubes containing serial dilutions of one or more previously purified bone marrow M-PC from individual patients (see *Online Supplementary Methods* for a more detailed description).

Allele-specific oligonucleotide polymerase chain reaction analysis of purified peripheral blood subpopulations of B cells and plasma cells

DNA was extracted from pre-defined numbers of highly-purified peripheral blood B-cell and PC subsets. ASO-PCR conditions were as described above; all purified cells from each cell compartment from each patient were analyzed by ASO-PCR (7 to 10 ASO-PCR replicates/patient). Clonotypic B cells were defined as being present within a specific B-cell or PC compartment when two or more replicates were found to be positive. For every sample, DNA quality was evaluated by a control according to the BIOMED-2 protocols.[32]

Results

Distribution of different subsets of B-lymphocytes and plasma cells in the peripheral blood of patients with multiple myeloma and monoclonal gammopathy or undetermined significance

The following subsets of peripheral blood B cells and PC were identified:[29,31] naïve ($CD10^-$ $CD19^+$ $CD20^+$ $CD27^-$ $CD38^-$) and memory ($CD10^-$ $CD19^+$ $CD20^+$ $CD27^+$ $CD38^-$) B-lymphocytes plus normal circulating PC ($CD10^-$ $CD19^+$ $CD20^-$ $CD27^{++}$ $CD38^{++}$). Peripheral blood memory B-lymphocytes were further subdivided into switched $SmIgA^+$, $_{Sm}IgG^+$, and non-switched $_{Sm}IgM^+$ cells. In four of the seven MM patients (cases #3, #5, #6 and #7), peripheral blood circulating M-PC with restricted CyIgλ or CyIgκ expression were also identified as clearly different from N-PC based on differential expression of CD38, CD19, CD45, and light scatter.[26,29]

Overall, no statistically significant differences ($P>0.05$, Mann-Whitney U test) were observed in the distribution of circulating B cells and PC between MM and MGUS patients (Figure 1). This was the case for total B cells (113±15 *versus* 83±71 cells/μL), naïve (83±11 *versus* 53±56 cells/μL) as well as total memory B cells (30±10 *versus* 22±16 cells/μL) and their $_{Sm}IgM+$ (9±4 *versus* 9±6 cells/μL), $_{Sm}IgG^+$ (14±2 *versus* 4±2 cells/μL), and $_{Sm}IgA+$ (12±1 *versus* 6±4 cells/μL) subsets, and also N-PC (0.7±0.4 *versus* 1.2±1.5 cells/μL) (Figure 1). In contrast, M-PC were found at frequencies >8.0 cells/μL (range: 0.08-29.8 cells/μL) in four of the seven MM patients, but not in the three MGUS cases (although we had detected them in up to 21% of a larger series of MGUS cases[16]) ($P<0.05$; Figure 1). Of note, all FACS-purified peripheral blood B-cell subsets (naïve and memory B-lymphocytes) and N-PC showed a normal protein expression profile, including a normal polyclonal Igκ *versus* Igλ light chain ratio, while the M-PC compartment systematically showed an aberrant immunophenotype associated with monoclonal light chain restriction (either $CyIg\kappa^+$ or $CyIg\lambda^+$) profile.

Table 1. Frequency and absolute counts of clonotypic cells detected in different B-cell and PC subsets present in the peripheral blood of patients with MGUS (n=3) or MM (n=7).

Case ID / Isotype of the M-Component	Subsets of B-lymphocytes						PC Subsets	
	Total B-lymphocytes	Naïve B-lymphocytes	Total memory B-lymphocytes	SmIgM⁺ memory B cells	SmIgG⁺ memory B cells	SmIgA⁺ memory B cells	N-PC	M-PC
MGUS 1 IgG⁺	<0.014 (<0.06%)	<0.004 (<0.007%)	<0.0105 (<0.05%)	<0.008 (<0.01%)	<0.015 (<0.2%)	<0.015 (<0.1%)	<0.0030 (<0.5%)	ND
MGUS 2 IgG⁺	<0.019 (<0.02%)	NT	<0.0064 (<0.01%)	<0.001 (<0.04%)	<0.009 (0.08%)	<0.008 (<0.08%)	<0.0058 (<0.5%)	ND
MGUS 3 IgG⁺	<0.018 (<0.02%)	NT	<0.0041 (<0.02%)	<0.002 (0.05%)	<0.011 (<0.2%)	<0.005 (<0.06%)	<0.00033 (<0.08%)	ND
MM 1 IgG⁺	<0.02 (<0.01%)	<0.017 (<0.1%)	<0.0078 (<0.04%)	<0.006 (0.06%)	<0.006 (<0.1%)	<0.048 (<0.4%)	<0.0031 (<0.8%)	ND
MM 2 IgA⁺	<0.03 (<0.03%)	<0.037 (<0.01%)	<0.0178 (<0.05%)	NT	NT	NT	NT	ND
MM 3 IgG⁺	<0.015 (<0.01%)	<0.007 (<0.01%)	<0.037 (<0.1%)	NT	<0.024 (<0.5%)	<0.011 (<0.2%)	<0.0042 (<0.6%)	29 (100%)
MM 4 IgA⁺	<0.03 (<0.11%)	<0.035 (<0.3%)	<0.026 (<0.3%)	NT	<0.012 (<0.6%)	<0.009 (<0.8%)	<0.00029 (<0.5%)	ND
MM5 IgG⁺	<0.03 (<0.03%)	<0.077 (<0.1%)	<0.013 (<0.05%)	<0.013 (<0.08%)	NT	NT	<0.024 (<14%)	0.7 (100%)
MM6 IgA⁺	<0.02 (<0.02%)	<0.025 (<0.02%)	<0.022 (<0.09%)	<0.034 (<0.2%)	NT	NT	<0.037 (<8%)	0.08 (100%)
MM7 IgG⁺	<0.006 (<0.01%)	<0.005 (<0.01%)	<0.01 (<0.03%)	<0.009 (<0.3%)	NT	NT	<0.013 (<0.2%)	6.1 (100%)

Results expressed as number of cells/μL and percentage of peripheral blood nucleated cells. The sensitivity of the assay was calculated based on the number of cells purified for each cell subset and its distribution in peripheral blood. ND: not detected; NT: not tested; MM: multiple myeloma; MGUS: monoclonal gammopathy of undetermined significance.

Sensitivity of the molecular assays

The sensitivity assays performed were aimed at defining the minimum number of clonotypic M-PC required for a positive result and at establishing the sensitivity of the ASO-PCR assay over a polyclonal background. The first set of experiments consistently showed the ability to systematically detect a (clonal) patient-specific *IGH* gene rearrangement peak whenever ≥5 clonotypic M-PC were present in the sample, for all ten patients analyzed; in three of the seven patients tested (cases #MM1, #MM2, #MGUS3) the sensitivity was even higher since patient-specific *IGH* CDR3 gene rearrangements were detected once a single M-PC was present in the sample. Regarding the ability to detect clonotypic M-PC over a background of polyclonal B cells, sensitivity from dilution experiments was systematically $<10^{-3}$, ranging between $<10^{-3}$ to $<10^{-5}$ (from 0.2% to 0.001% M-PC cells/total B cells) (*Online Supplementary Table S2*), as illustrated in Figure 3 for one of the cases studied.

The sensitivity of the assay varied per patient for the different cell fractions from <0.08 to <0.0003 cells/μL of peripheral blood (i.e. <80 to <0.3 cells/mL), according to: the sensitivity and reproducibility of the assay; the number of purified peripheral blood B cells and PC analyzed; and the distribution of the subpopulation (e.g. absolute count) in the peripheral blood of each individual patient at the moment the sample was collected (Table 1).

Frequency of clonotypic cells in highly-purified peripheral blood subsets of circulating B cells and plasma cells

The search for the presence of clonotypic cells was performed on highly purified peripheral blood naïve B-lymphocytes, switched $_{Sm}$IgA⁺ and $_{Sm}$IgG⁺, as well as non-switched $_{Sm}$IgM⁺ memory B cells and N-PC, present at variable numbers in the peripheral blood of each of the ten patients with monoclonal gammopathies (Table 1). In addition, circulating M-PC from four MM patients in whom these cells were detected at frequencies of 0.79%, 0.09%, 0.03% and 0.17% of all peripheral blood nucleated cells (absolute M-PC count of 29.8, 0.7, 0.08, and 6.1 cells/μL, respectively) were also purified and analyzed. Overall, no cells carrying clonotypic *IGH* gene rearrangements were detected among the phenotypically normal B-cell subsets or N-PC from any of the MM (0/7) or MGUS (0/3) patients analyzed (Table 1). For Ig-switched memory B cells sharing the same Ig heavy chain isotype as bone marrow M-PC, a sensitivity of between <6 cells/mL and <24 cells/mL of peripheral blood (IgG⁺ MM) and of <9 cells/mL (IgA⁺ MM) of peripheral blood was reached. As expected, analysis of peripheral blood M-PC confirmed the presence of clonotypic *IGH* gene rearrangements identical to those identified in the corresponding (paired) bone marrow M-PC, in all replicates of purified M-PC analyzed (Table 1 and Figure 2).

Discussion

Since the pioneering experiments which used anti-idiotypic antibodies to track the oncogenic event in MM throughout B-cell ontogeny,[36,37] controversial results have been reported in the literature regarding the B-cell compartment in which the potential myeloma stem cell resides. For more than a decade now, it has been generally accepted that circulating CD19⁺ B-lymphocytes from MM patients could contain cells that carry clonotypic rearrangements identical to those of MM-PC.[2-7] However, the frequency and nature of such clonally-related cells remains a matter of debate.

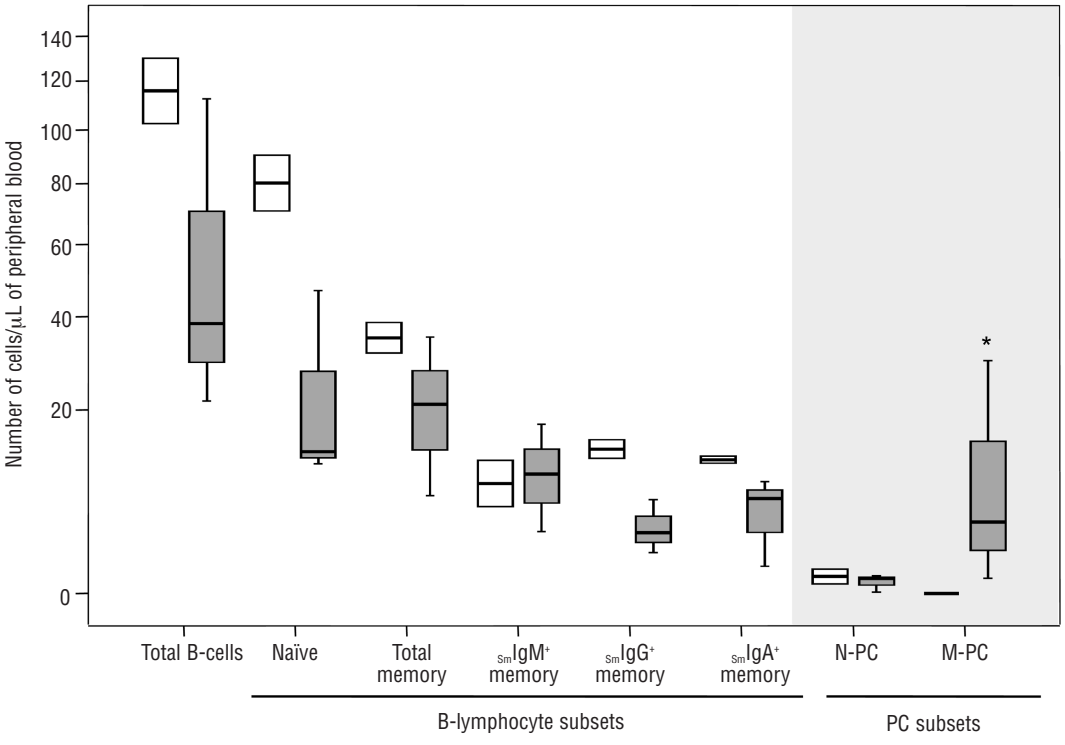

Figure 1. Distribution of B-lymphocytes and plasma cells and their subsets in the peripheral blood of patients with MGUS (n=3, white boxes) and MM (n=7; black boxes). N-PC: normal plasma cells; M-PC: (mono)clonal plasma cells. Boxes extend from the 25th to the 75th percentiles; the line in the middle and the vertical lines represent median values and both the 10th and 90th percentiles, respectively. The Mann–Whitney U test was used to estimate the statistical significance of differences observed. **P*<0.05 *vs.* MGUS.

While some groups suggested that the majority of circulating B-lymphocytes would belong to the malignant clone,[2,3] others could not confirm these preliminary observations and reported that, if present, circulating clonotypic B cells would constitute a minor population of all peripheral blood leukocytes.[38,39]

Important advances have been achieved in molecular as well as in cell purification techniques (e.g. improved exclusion of cell doublets and sorting efficiency, standard usage of ≥8-color experiments for better definition of the cell subpopulations of interest and exclusion of unwanted cells). Based on such technological advances we decided to revisit in the present study the detection of clonally related B cells in the peripheral blood of patients with monoclonal gammopathies. For this purpose we used a highly-sensitive approach. First, an eight-color multiparameter flow cytometry technique was used for the identification of the distinct subsets of circulating peripheral blood B cells and PC, in combination with multiparameter sorting of highly purified, non-contaminated, cell fractions corresponding to such subsets of peripheral blood naïve and memory B-lymphocytes (including $_{Sm}IgM^+$, $_{Sm}IgA^+$ and $_{Sm}IgG^+$ memory B cells), in addition to N-PC and, whenever present, also M-PC. In a second step, molecular detection of clonotypic B cells was performed on DNA from the purified B-cell subsets and PC using a highly-sensitive ASO-PCR technique specifically designed for the detection of clonotypic B cells of individual patients.

Our results showed systematic absence of clonotypic *IGH* gene rearrangements in all different B-cell subsets analyzed, including M-component isotype-matched memory B-lymphocytes; the only exception were the M-PC detected and purified from the peripheral blood of four MM patients. In line with these observations, no altered patterns of protein expression were found in any of the purified fractions of naïve and memory B-lymphocytes or N-PC, all such cell subsets also showing a normal polyclonal Igκ *versus* Igλ light chain ratio, except M-PC.

Considering the sensitivity of the assay used, the number of cells analyzed and the volume of blood investigated, it seems unlikely that circulating clonotypic B-lymphocytes are present in MM and MGUS patients at frequencies between >6 and 30 cells/mL (<1 cell in between 33 and 166 μL of peripheral blood). Even more, when we specifically consider the absence of clonotypic B cells among the compartment of memory B cells (as the most susceptible to containing the myeloma stem cell) the frequency would go down to between <4.1 and <37 cells/mL.

Overall, the sensitivity of the approach used here is far beyond that of the techniques used in most previously reported studies in which the presence of clonotypic B-lymphocytes was reported. In this regard, the discrepancies observed between our and other studies cannot be due to selection of patients[12] since almost all our MM patients had symptomatic MM and they had not received prior therapy for their disease. We did confirm the presence of circulating M-PC at relatively high numbers in the peripheral blood of the majority of MM patients (range: 80 to 29,800 cells/mL), in line with previous observations by our and other groups.[12-16] This frequency is lower than that reported in earlier studies for circulating clonotypic B cells (range: 10,000 to 610,000 cells/mL).[3] It is worth noting that, in a substantial number of these previous studies, total peripheral blood B cells were used to search for clonotypic *IGH* gene rearrangements and contamination by M-PC (e.g. due to doublet formation or altered time delay in sorting experiments) was not systematically excluded. In this regard, we have recently confirmed contamination of peripheral blood

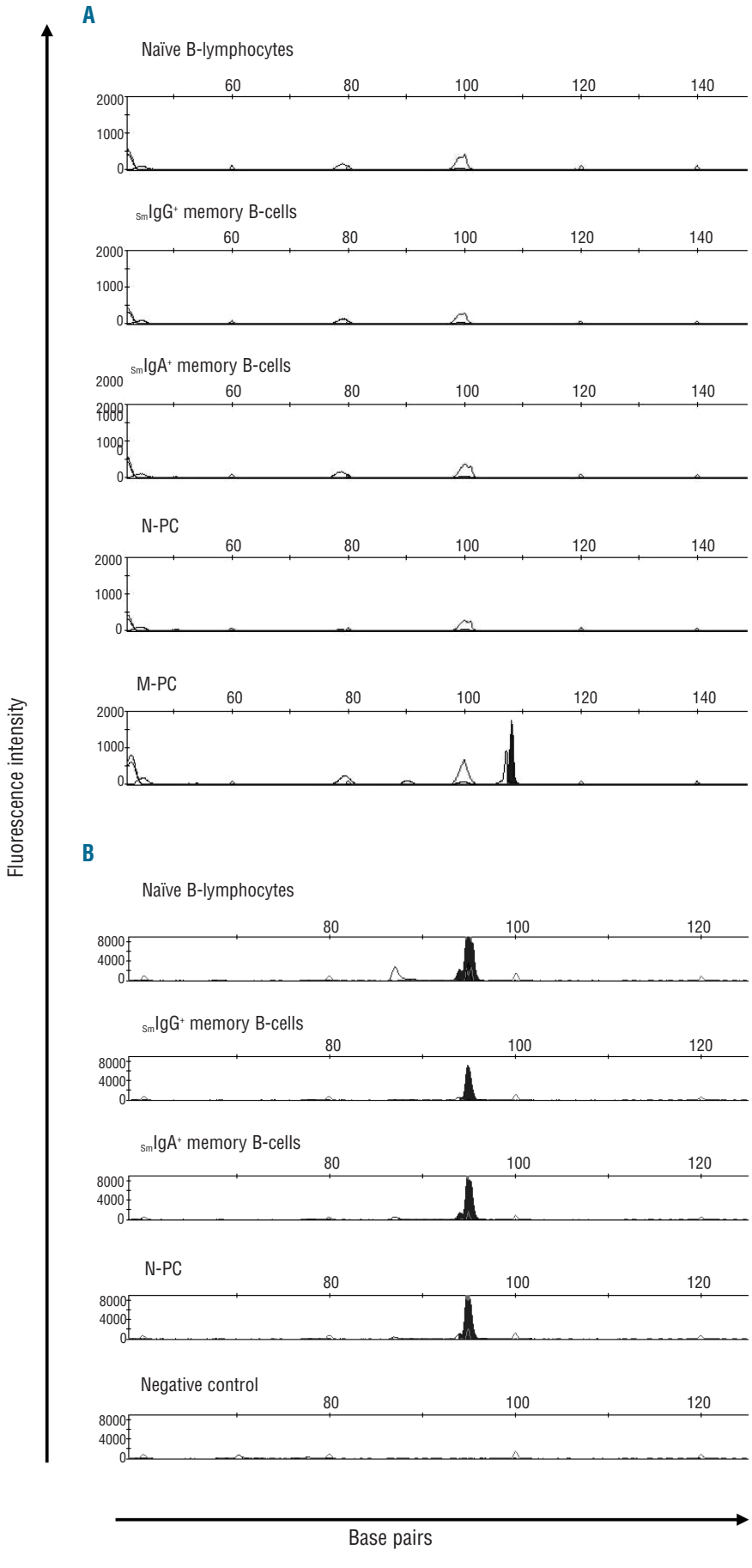

Figure 2. Example of the results of molecular analysis of clonotypic B cells and PC in purified B-cell and PC subsets from the peripheral blood (PB) of a MM patient (case #MM3). As shown, circulating (mono)clonal plasma cells (M-PC) are the only compartment of PB B cells from this patient that showed a clonal *CDR3* gene rearrangement signature similar to that of bone marrow M-PC from the same patient (A). The quality of the DNA samples from all FACS-purified PB B-cell and PC subsets of each individual patient was analyzed in parallel for a control gene with PCR primers directed to the human thromboxane synthase gene (*TBXAS1*) (B).

B cells by M-PC through re-analysis of a frozen sample from a patient who had been previously reported to carry clonotypic non-switched B cells.[26] This could contribute to explain, at least in part, the discrepancies observed between our and other studies, even when our approach dramatically increased the sensitivity and specificity of the analysis. In contrast, our results fully support recent findings by Kim *et al.*[21] in SCID mice previously engrafted subcutaneously with fetal human bone, showing that only the MM-PC compartment was able to give rise to clonally-related, patient-specific MM cells in (two) serial transplants; although plasmablasts and B cells were also able to engraft in those mice, their capacity was limited to the first xeno-transplantation and these compartments could not give rise to any cell with the same patient-specific MM molecular signature.[21] Alternatively, it is possible that the tumor precursor would circulate only at the earlier stages of disease to fill/feed the bone marrow niches, giving rise to tumor PC. However, this seems unlikely since different peripheral blood B-cell compartments from MGUS patients analyzed here were also found to be devoid of clonotypic cells with the same *IGH* gene rearrangement as bone marrow M-PC.

Altogether, our results suggest that if peripheral blood clonal B-lymphocytes exist in MM and MGUS, they are present at extremely low frequencies in most patients, and would not explain the dissemination throughout the bone marrow during active/progressive disease, as observed in many symptomatic MM patients. Conversely, since circu-

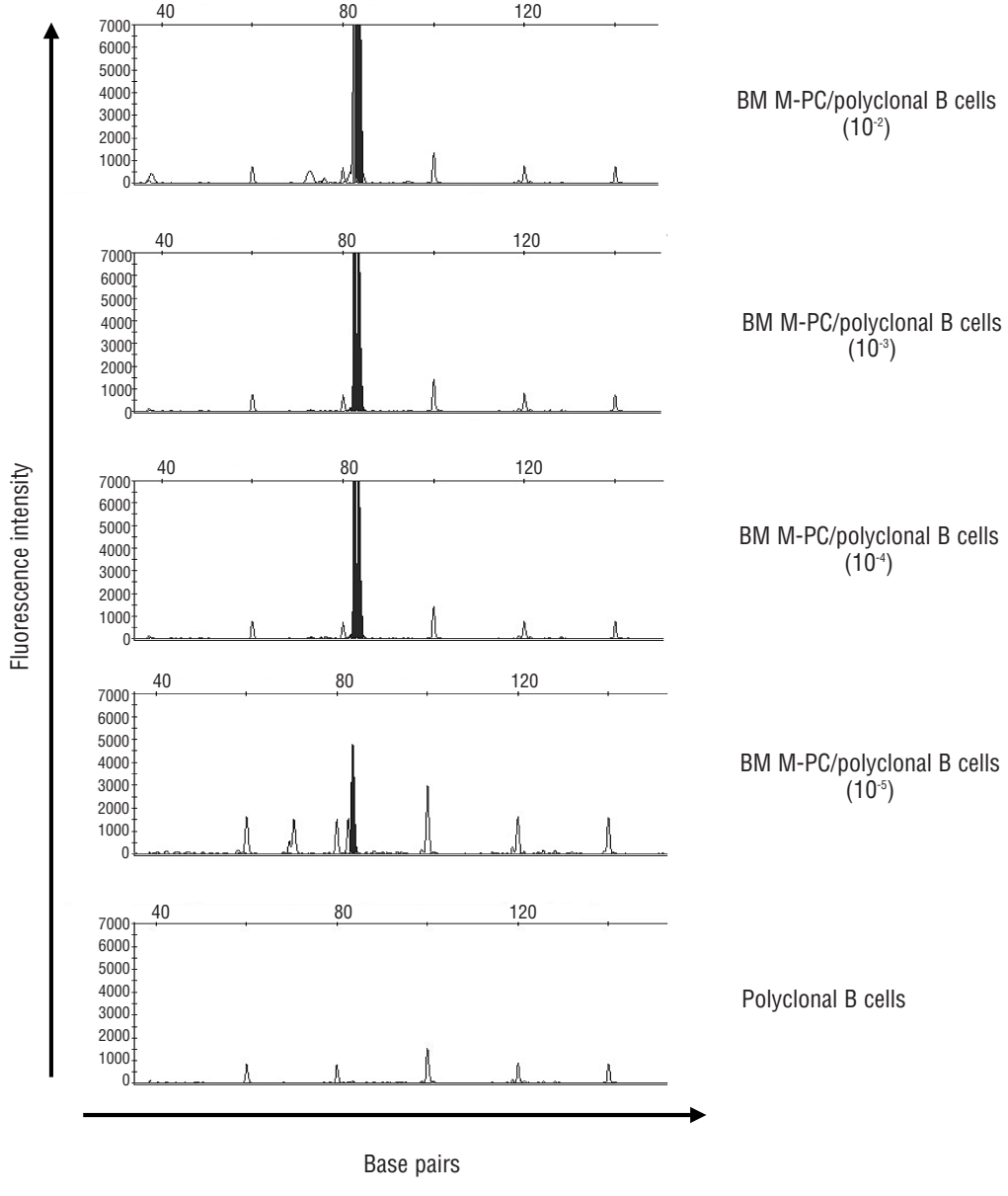

Figure 3. Example of a dilution experiment performed and analyzed by GeneScanning for one representative MM patient (case #MM6). To evaluate the sensitivity of the ASO-PCR assay for this patient, DNA from M-PC obtained from the bone marrow (BM) was diluted into polyclonal DNA obtained from FACS-purified peripheral blood (PB) B cells from a healthy donor. Numbers between brackets indicate the M-PC:normal B-cell dilution factor.

lating M-PC were frequently detected at rather higher numbers, it could be hypothesized that the myeloma clonogenic cell could be part of the malignant PC compartment which is able to re-circulate and spread the disease. In line with this latter hypothesis recent findings indicate that circulating peripheral blood M-PC from both MM and MGUS patients display a slightly more immature immunophenotype with lower expression of CD138,[16] a phenotypic feature of M-PC that has been associated with increased clonogenic potential and other myeloma stem cell features.[4,19] Furthermore, circulating peripheral blood M-PC are also frequently detected in early phases of the disease including MGUS and smoldering MM.[12,16] Further studies, in which the clonogenic potential of peripheral blood M-PC is compared with that of bone marrow tumor cells, are required to validate this hypothesis.

In summary, based on a sensitive and well-controlled approach, in this study we failed to demonstrate the presence of circulating clonotypic B cells in the peripheral blood of patients with MM or MGUS, outside the compartment of immunophenotypically aberrant clonal PC detected in a substantial fraction of MM patients. These findings suggest that tumor dissemination in monoclonal gammopathies is more likely to be related to the peripheral blood compartment of circulating M-PC than to other populations of less differentiated naïve and memory B-lymphocytes.

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References

1. San Miguel JF, García-Sanz R, González M, Moro MJ, Hernández JM, Ortega F, et al. A new staging system for multiple myeloma based on the number of S-phase plasma cells. Blood. 1995;85(2):448-55.
2. Bergsagel PL, Masellis Smith A, Belch AR, Pilarski LM. The blood B-cells and bone marrow plasma cells in patients with multiple myeloma share identical IgH rearrangements. Curr Top Microbiol Immunol. 1995;194:17-24.
3. Szczepek AJ, Seeberger K, Wizniak J, Mant MJ, Belch AR, Pilarski LM. A high frequency of circulating B cells share clonotypic Ig heavy-chain VDJ rearrangements with autologous bone marrow plasma cells in multiple myeloma, as measured by single-cell and in situ reverse transcriptase-polymerase chain reaction. Blood. 1998;92(8): 2844-55.
4. Pilarski LM, Hipperson G, Seeberger K, Pruski E, Coupland RW, Belch AR. Myeloma progenitors in the blood of patients with aggressive or minimal disease: engraftment and self-renewal of primary human myeloma in the bone marrow of NOD SCID mice. Blood. 2000;95(3):1056-65.
5. Matsui W, Huff CA, Wang Q, Malehorn MT, Barber J, Tanhehco Y, et al. Characterization of clonogenic multiple myeloma cells. Blood. 2004;103(6):2332-6.
6. Rasmussen T, Haaber J, Dahl IM, Knudsen LM, Kerndrup GB, Lodahl M, et al. Identification of translocation products but not K-RAS mutations in memory B cells from patients with multiple myeloma. Haematologica. 2010;95(10):1730-7.
7. Boucher K, Parquet N, Widen R, Shain K, Baz R, Alsina M, et al. Stemness of B-cell progenitors in multiple myeloma bone marrow. Clin Cancer Res. 2012;18(22): 6155-68.
8. Zojer N, Schuster-Kolbe J, Assmann I, Ackermann J, Strasser K, Hübl W, et al. Chromosomal aberrations are shared by malignant plasma cells and a small fraction of circulating CD19+ cells in patients with myeloma and monoclonal gammopathy of undetermined significance. Br J Haematol. 2002;117(4):852-9.
9. Sahota SS, Leo R, Hamblin TJ, Stevenson FK. Ig VH gene mutational patterns indicate different tumor cell status in human myeloma and monoclonal gammopathy of undetermined significance. Blood. 1996;87 (2):746-55.
10. Schmidt-Hieber M, Gutiérrez ML, Pérez-Andrés M, Paiva B, Rasillo A, Tabernero MD, et al. Cytogenetic profiles in multiple myeloma and monoclonal gammopathy of undetermined significance: a study in highly purified aberrant plasma cells. Haematologica. 2013;98(2):279-87.
11. Walker BA, Wardell CP, Melchor L, Hulkki S, Potter NE, Johnson DC, et al. Intraclonal heterogeneity and distinct molecular mechanisms characterize the development of t(4;14) and t(11;14) myeloma. Blood. 2012;120(5):1077-86.
12. Billadeau D, Van Ness B, Kimlinger T, Kyle RA, Therneau TM, Greipp PR, et al. Clonal circulating cells are common in plasma cell proliferative disorders: a comparison of monoclonal gammopathy of undetermined significance, smoldering multiple myeloma, and active myeloma. Blood. 1996; 88(1):289-96.
13. Schneider U, van Lessen A, Huhn D, Serke S. Two subsets of peripheral blood plasma cells defined by differential expression of CD45 antigen. Br J Haematol. 1997;97(1): 56-64.
14. Rawstron AC, Owen RG, Davies FE, Johnson RJ, Jones RA, Richards SJ, et al. Circulating plasma cells in multiple myeloma: characterization and correlation with disease stage. Br J Haematol. 1997;97(1):46-55.
15. Luque R, Brieva JA, Moreno A, Manzanal A, Escribano L, Villarrubia J, et al. Normal and clonal B lineage cells can be distinguished by their differential expression of B cell antigens and adhesion molecules in peripheral blood from multiple myeloma (MM) patients-diagnostic and clinical implications. Clin Exp Immunol. 1998;112 (3):410-8.
16. Paiva B, Pérez-Andrés M, Vídriales MB, Almeida J, de las Heras N, Mateos MV, et al. Competition between clonal plasma cells and normal cells for potentially overlapping bone marrow niches is associated with a progressively altered cellular distribution in MGUS vs myeloma. Leukemia 2011;25(4):697-706.
17. Jakubikova J, Adamia S, Kost-Alimova M, Klippel S, Cervi D, Daley JF, et al. Lenalidomide targets clonogenic side population in multiple myeloma: pathophysiologic and clinical implications. Blood. 2011;117(17):4409-19.
18. Bakkus MH, Heirman C, Van Riet I, Van Camp B, Thielemans K. Evidence that multiple myeloma Ig heavy chain VDJ genes contain somatic mutations but show no intraclonal variation. Blood. 1992;80(9): 2326-35.
19. Matsui W, Wang Q, Barber JP, Brennan S, Smith BD, Borrello I, et al. Clonogenic multiple myeloma progenitors, stem cell properties, and drug resistance. Cancer Res. 2008;68(1):190-7.
20. Paino T, Ocio EM, Paiva B, San-Segundo L, Garayoa M, Gutierrez NC, et al. CD20 positive cells are undetectable in the majority of multiple myeloma cell lines and are not associated with a cancer stem cell phenotype. Haematologica. 2012;97(7):1110-4.
21. Kim D, Park CY, Medeiros BC, Weissman IL. CD19-CD45 low/- CD38 high/CD138+ plasma cells enrich for human tumorigenic myeloma cells. Leukemia. 2012;26(12): 2530-7.
22. Yaccoby S, Epstein J. The proliferative potential of myeloma plasma cells manifest in the SCID-hu host. Blood. 1999;94(10): 3576-82.
23. Yata K, Yaccoby S. The SCID-rab model: a novel in vivo system for primary human

myeloma demonstrating growth of CD138-expressing malignant cells. Leukemia. 2004;18(11):1891-7.
24. Musto P, Carella AM Jr, Greco MM, Falcone A, Sanpaolo G, Bodenizza C, et al. Short progression-free survival in myeloma patients receiving rituximab as maintenance therapy after autologous transplantation. Br J Haematol. 2003(4);123:746-7.
25. Zojer N, Kirchbacher K, Vesely M, Hübl W, Ludwig H. Rituximab treatment provides no clinical benefit in patients with pretreated advanced multiple myeloma. Leuk Lymphoma. 2006;47(6):1103-9.
26. Pfeifer S, Perez-Andres M, Ludwig H, Sahota SS, Zojer N. Evaluating the clonal hierarchy in light-chain multiple myeloma: implications against the myeloma stem cell hypothesis. Leukemia. 2011;25(7):1213-6.
27. The International Myeloma Working Group. Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the International Myeloma Working Group. B J Haematol. 2003;121(5):749-57.
28. Kalina T, Flores-Montero J, van der Velden VH, Martin-Ayuso M, Böttcher S, Ritgen M, et al. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. Leukemia 2012;26(9):1986-2010.
29. Caraux A, Klein B, Paiva B, Bret C, Schmitz A, Fuhler GM, et al. Circulating human B and plasma cells. Age-associated changes in counts and detailed characterization of circulating normal CD138- and CD138+ plasma cells. Haematologica 2010;95(6):1016-20.
30. Paiva B, Gutiérrez NC, Chen X, Vídriales MB, Montalbán MÁ, Rosiñol L, et al; Clinical significance of CD81 expression by clonal plasma cells in high-risk smoldering and symptomatic multiple myeloma patients. Leukemia. 2012;26(8):1862-9.
31. Perez-Andres M, Paiva B, Nieto WG, Caraux A, Schmitz A, Almeida J, et al. Human peripheral blood B-cell compartments: a crossroad in B-cell traffic. Cytometry B Clin Cytom. 2010;78 (Suppl 1):S47-60.
32. van Dongen JJ, Langerak AW, Brüggemann M, Evans PA, Hummel M, Lavender FL, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003;17(12):2257-317.
33. Langerak AW, Groenen PJ, Bruggemann M, Beldjord K, Bellan C, Bonello L, et al. EuroClonality/BIOMED-2 guidelines for interpretation and reporting of Ig/TCR clonality testing in suspected lymphoproliferations. Leukemia. 2012;26(10):2159-71.
34. Puig N, Sarasquete ME, Alcoceba M, Balanzategui A, Chillon MC, Sebastian E, et al. The use of CD138 positively selected marrow samples increases the applicability of minimal residual disease assessment by PCR in patients with multiple myeloma. Ann.Hematol. 2013;92(1):97-100.
35. Sarasquete ME, García-Sanz R, González D, Martínez J, Mateo G, Martínez P, et al. Minimal residual disease monitoring in multiple myeloma: a comparison between allelic-specific oligonucleotide real-time quantitative polymerase chain reaction and flow cytometry. Haematologica. 2005(10); 90:1365-72.
36. Mellstedt H, Hammarström S, Holm G. Monoclonal lymphocyte population in human plasma cell myeloma. Clin Exp Immunol. 1974(3);17:371-84.
37. Kubagawa H, Vogler LB, Capra JD, Conrad ME, Lawton AR, Cooper MD. Studies on the clonal origin of multiple myeloma. Use of individually specific (idiotype) antibodies to trace the oncogenic event to its earliest point of expression in B-cell differentiation. J Exp Med. 1979(4);150:792-807.
38. Chen BJ, Epstein J. Circulating clonal lymphocytes in myeloma constitute a minor subpopulation of B cells. Blood. 1996;87(5): 1972-6.
39. Joshua DE, Brown RD, Luo XF, Gibson J. Circulating clonal lymphocytes in myeloma determined by mRNA in situ hybridization. Blood. 1996;88(3):1125.